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Attorney Docket No.: 10609.013-US

PATENT

PROVISIONAL APPLICATION FOR PATENT COVER SHEET

Commissioner for Patents
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Sir:

This is a request for filing a provisional application under 37 CFR 1.53(c).

INVENTOR(S)		
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TITLE OF THE INVENTION		
A method to get increased yield of lactobionic acid		

The following application parts are enclosed:

[x] specification 19 pages [] Sequence Listing __ pages

[x] Abstract 1 page [x] Drawings 1 page

An application data sheet is enclosed.

Direct all correspondence to Customer Number 25908.

Please charge the required fee, estimated to be $160, to Novozymes North America, Inc., Deposit Account No. 50-1701. A duplicate of this sheet is enclosed.

Respectfully submitted,

Date: July 1, 2004

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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

EXPRESS MAIL CERTIFICATE

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Re: U.S. Provisional Application for
"A method to get increased yield of lactobionic acid"
Applicants: Nielsen, et al.

Sir:

Express Mail Label No. EV 371426554 US

Date of Deposit July 1, 2004

I hereby certify that the following attached paper(s) or fee

1. Filing Under 37 C.F.R. §1.53(c) (in duplicate)
2. Provisional Application
3. Application Data Sheet

are being deposited with the United States Postal Service "Express Mail Post Office to Addressee" under 37 C.F.R. 1.10 on the date indicated above and is addressed to the address indicated above.

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Application Data Sheet

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Initial 07/01/04

TITLE: A method to get increased yield of lactobionic acid.

FIELD OF THE INVENTION:

A method for getting increased yields of lactobionic acid comprising adding to a dairy base, such as milk or whey, a carbohydrate oxidase, capable of converting lactose to lactobionic acid, wherein the method is performed under stably control of pH from pH 5 to 6.9.

BACKGROUND OF THE INVENTION:

Lactose, commonly called milk sugar, is the primary carbohydrate of milk. In milk based dairy products such as yoghurt, cheese and milk lactose may be considered a low value sugar because of e.g. lactose intolerance and due to its contribution to browning reactions and crystallisation.

Carbohydrate oxidase enzymes capable of converting lactose to lactobionic acid are known. The reaction scheme may be described as:

$$\text{lactose} + \tfrac{1}{2}O_2 \rightarrow \text{lactobionic acid} + H_2O_2$$

See figure 1 for further details. The CAS reg. no. for lactobionic acid is 96-82-2.

WO02/089592 (Kraft Foods) describes a number of advantages with respect to use a lactobionic acid in milk based dairy products and advantages with respect of carbohydrate oxidase based enzymatic *in situ* conversion of lactose into lactobionic acid during the process of preparing milk based dairy products.

The advantages relates e.g. to making products with less lactose (e.g. milk with less lactose) or processed cheese products (e.g. for pizza) that have less browning problems. Further, in e.g. cheese production lactobionic acid may be used to develop acidity. Generally acidity is developed by fermenting milk with lactic acid bacteria that metabolize lactose to produce lactic acid. Consequently by use of lactobionic acid one may produce relevant products by using less amounts of lactic acid bacteria.

With respect to the carbohydrate oxidase based enzymatic conversion of lactose into lactobionic acid, WO02/089592 essentially describes that the enzyme is to be added to a dairy base (e.g.

milk or whey) and then incubated for a certain period of time at a suitable temperature in order to accomplish the enzyme reaction. The description provides no explicit teaching with respect to any advantages of maintaining the pH at a certain level during the enzymatic reaction. If pH is not controlled the pH will decrease during the reaction due to the generation of the lactobionic acid.

In example 9 of WO02/089592 milk is incubated with the enzyme oxidase overnight. No reference is made by any control of pH. After the overnight incubation, the treated milk comprised 1.5% lactose and 3.2% lactobionic acid, which causes a conversion of lactose to lactobionic acid of 68%.

In example 12 B) and 12 C) milk is incubated with the oxidase for 48 hours at 55°C. During the reaction the pH is maintained at pH 7. No explanation is given for this pH control and no lactobionic acid yields are provided in these examples.

WO03/037093 (Novozymes) also describes a carbohydrate oxidase based enzymatic conversion of lactose into lactobionic acid during the process of preparing milk based dairy products. As for WO02/089592, this document is also silent with respect to any advantages of maintaining the pH at a certain level during the enzymatic reaction. The only specific working example describes that full fat milk was incubated with the oxidase and allowed to react at 40°C until pH 4.2 was reached. It thus appears that pH was not controlled as pH of natural fresh milk is approximately 6.6.

WO02/39828 (Danisco) relates to a process where a hexose oxidase solution is sprayed onto a pizza with cheese and the advantageous effect of less browning (termed "Maillard reaction") of the pizza cheese is demonstrated. This document is also silent with respect to any advantages of maintaining the pH at a certain level during the enzymatic reaction.

SUMMARY OF THE INVENTION:

The problem to be solved by the present invention is to provide a method to get higher yields of lactobionic acid from a dairy base.

The solution is based on the observation by the present inventors that by stably maintaining the pH to a value from 5 to 6.9 during the carbohydrate oxidase based enzymatic conversion of lactose into lactobionic acid higher yields of lactobionic acid than described in the art are achieved.

Accordingly, a first aspect of the invention relates to a method for the preparation of a composition comprising lactobionic acid comprising

(i) adding to a dairy base a carbohydrate oxidase, capable of converting lactose to lactobionic acid,

(ii) incubating for a suitable time at a suitable temperature in presence of a suitable amount of oxygen to obtain the composition comprising lactobionic acid, and

(iii) optionally, purifying the lactobionic acid in a suitable way to get a composition comprising lactobionic acid with a desired degree of lactobionic acid purity,

characterized in that during the incubating the pH is maintained, by adequate addition of a base, at a pH from 5 to 6.9.

Preferably, the adequate addition of a base is done for a time period that is sufficient long to get a degree of conversion of lactose to lactobionic acid that is at least 2.5% higher than in a comparative control method where the only comparative difference is that during the incubation the pH is not maintained by adequate addition of a base.

For illustration, in working examples herein is shown that the degree of conversion of lactose to lactobionic acid of the control sample was 41% and by maintaining pH stable as described herein the degree of conversion increased to 90%.

If the dairy base is for instance milk or whey the resulting milk or whey composition with higher yield of lactobionic acid may then advantageously be used to make a dairy product such as cheese, yoghurt or milk. Numerous suitable uses of a composition comprising lactobionic acid as described herein to make a dairy product of interest is known to the skilled person and reference is e.g. made to the prior art publications discussed above.

Accordingly, a second aspect of the invention relates to a process for making a dairy product comprising first to make a composition comprising lactobionic acid according to a method for

making such a composition as described herein and then use this composition to make the dairy product.

DRAWINGS

Figure 1 shows a reaction scheme for a carbohydrate oxidase enzyme conversion of lactose to lactobionic acid.

DETAILED DESCRIPTION OF THE INVENTION:

Dairy base:

The term "dairy base" is to be understood as any milk or milk like product including lactose, such as whole or low fat milk, skim milk, buttermilk, condensed milk, dried milk, whey, whey permeate, lactose, mother liquid from crystallization of lactose, whey protein concentrate, or cream originating from any animal.

Preferably the dairy base is milk and more preferably whey or fractions of whey.

The term "Milk" is here to be understood as the lacteal secretion obtained by milking any mammal, such as cows, sheep, goats, buffaloes or camels.

The present method is especially suitable for relatively large scale production. Accordingly in a preferred embodiment is used from 50 kg to 500000 kg of the dairy base in the method as described herein.

Lactobionic acid:

It is to be understood that the term "lactobionic acid" relates to that the end-product can be lactobionic acid or salts thereof. Suitable salt include Na-lactobionate, Ca-lactobionate and K- lactobionate.

The base used for pH control is selected to control the type of lactobionate produced.

Carbohydrate oxidase:

A number of suitable carbohydrate oxidases, capable of converting lactose to lactobionic acid, are known and available to the skilled person. It may for instance be a hexose oxidase or a glucose oxidase.

Preferably, the carbohydrate oxidase is a microbial carbohydrate oxidase.

A suitable hexose oxidase (EC1.1.3.5) is described in WO96/40935 (Bioteknologisk Institut, Denmark). This document describes a suitable hexose oxidase from marine algal species more particular wherein the marine algal species is one selected from the group consisting of *Chondrus crispus*, *Iridophycus flaccidum* and *Euthora cristata*.

Other suitable carbohydrate oxidase may be derived, e.g., from a mitosporic Pyrenomycetes such as *Acremonium*, in particular, *A. strictum*, such as ATCC 34717 or T1; *A. fusidioides*, such as IFO 6813; or *A.potronii*, such as IFO 31197. In a preferred embodiment, the carbohydrate oxidase is obtained from the source disclosed by Lin, et al, (1991, Biochim. Biophys. Acta 1118:41-47) and in JP-A 5-84074.

In a preferred embodiment the carbohydrate oxidase is a carbohydrate oxidase obtained from a fungus belonging to the genus *Microdochium*, more preferably wherein the fungus is *Microdochium nivale* and even more preferably wherein the fungus is *Microdochium nivale* CBS 100236. Such a preferred oxidase is described in details in WO99/31990 (Novo Nordisk A/S).

The amount of oxidase to be used will generally depend on the specific needs. Preferably, an amount of oxidase is used that is from 0.1 to 1000 OXU per kg of dairy base, more preferably from 1 to 500 OXU per kg of dairy base, and even more preferably from 5 to 100 OXU per kg of dairy base.

An Oxidase Unit (OXU) is herein basically defined as the amount of enzyme that oxidizes one µmol lactose per minute under the incubation conditions of the method one specifically uses.

Incubating under suitable conditions:

Incubation according to step (ii) of the method as described herein should be for a suitable time at a suitable temperature in presence of a suitable amount of oxygen to obtain the composition comprising lactobionic acid. It is within the general knowledge of the skilled person to determine such suitable conditions in relation to the specific elements of a specific method of interest.

Generally, a suitable time should be a time period that is long enough to obtain the degree of conversion of lactose to lactobionic of interest.

The conversion of lactose to lactobionic consumes oxygen. This can e.g. be seen in the table of example 1 herein. Accordingly, if the oxygen is monitored during the enzymatic reaction one will generally see an initial drop in oxygen amount which, if e.g. air is constantly provided, will return to around the initial level when the enzyme reaction terminates. When oxygen has returned to more than 90% of the initial level it is an indication for that the enzymatic reaction has ended or at least been significantly slowed down. Accordingly, a suitable time period could preferably be for a time period that at least last until the oxygen level of the incubated dairy base has returned to more than 90% of the initial level. This is especially if one wants maximum conversion of lactose.

Generally a suitable time period is from ½ hours to 3 days, more preferably from 2 hours to 24 hours, even more preferably from 2 hours to 12 hours.

A suitable temperature will generally depend on the oxidase used and is generally optimized according the optimal reaction temperature for the oxidase. Generally, a suitable temperature will be form 0°C to 80°C. Low temperature, as for instance 5°C, results in a relatively slow reaction rate, but has the advantage of representing the typical temperature for cold stored products.

A suitable amount of oxygen may for instance be obtained by continuously mixing air into the dairy base under incubation. The skilled person knows how to optimize this.

Incubating at a stable pH:

As explained above, an important element of the method as described herein is that during the incubating (of step (ii)) the pH is to be maintained, by adequate addition of a base, at a pH from 5 to 6.9 for a time period that is sufficient long to get a degree of conversion of lactose to lactobionic acid that is at least 2.5% higher than in a comparative control method where the only comparative difference is that during the incubation the pH is not maintained by adequate addition of a base.

The comparative control method should, except for the non addition of the base to maintain the pH, be performed identical (same oxidase in same amount, same dairy base, same incubation time, temperature and presence of oxygen and etc.) to the method for improving lactobionic acid as described herein. This is in accordance with the normal understanding of a control as used herein, because the control is made to identify the positive effect of maintaining pH stable.

The preferred pH value for a specific method of interest will, as known to the skilled person, depend on a number of factors. For instance, if dairy base is milk the natural pH of milk is known to be around 6.6 and in such a case it would be preferred to maintain the pH in a value around 6.6, such as a pH from 6.3 to 6.9.

The base may be any suitable base such as an adequate NaOH solution. The skilled person knows numerous of other suitable bases. Examples are $Ca(OH)_2$, KOH, NH_4OH, $Mg(OH)_2$ and Na-tripoly-phosphate.

The preferred base will generally be a base that relates to the preferred mineral composition of the final dairy product.

For instance, in particular when the diary base is milk, a preferred base may be $Ca(OH)_2$. This is in particular preferred if a final dairy product is desired, which is enriched with calcium. As illustrated in a work herein, the method as described herein may be used to make a milk product enriched with calcium.

When the base is e.g. $Ca(OH)_2$ it is possible by using the method as described herein, to make Ca–lactobionate from a suitable dairy base. This product can then be used as e.g. a dairy product additive or an ingredient as known in the art.

Preferably, during the incubating (of step (ii)) the pH is maintained, by adequate addition of a base, at a pH from 5 to 6.9 for a time period that is sufficient long to get a degree of conversion of lactose to lactobionic acid that is at least 5% higher than in a comparative control method where the only comparative difference is that during the incubation the pH is not maintained by adequate addition of a base, more preferably at least 15% higher than in the comparative control method, even more preferably at least 30% higher than in the comparative control method and most preferably at least 45% higher than in the comparative control method.

For illustration, in working examples herein is shown that the degree of conversion of lactose to lactobionic acid of the control was 41% and by maintaining pH as described herein the degree of conversion increased to 90%.

Preferably the pH is maintained, by adequate addition of a base, at a pH from 5.5 to 6.9, more preferably at a pH from 6 to 6.9.

Preferably, the pH is maintained at the stable pH level as described herein from the start of the enzymatic reaction. In other words, immediately after the oxidase is added to the dairy base the base is added to maintain the pH as described herein.

Especially if one wants maximum conversion of lactose it is preferred that the pH is maintained at the stable pH level as described herein for a time period that at least last until the oxygen level of the incubated dairy base has returned to more than 90% of the initial level.

Preferably, the pH is maintained at the stable pH level as described herein for a time period from 30 minutes to 35 hours, more preferably from 1 hour to 20 hours and even more preferably from 2 hours to 12 hours.

Catalase:

A catalase is an enzyme that catalyses the reaction: $2\ H_2O_2 \Rightarrow O_2 + 2\ H_2O$. The EC number is EC 1.11.1.6.

As shown in working example 3 herein use of a Catalase significantly improves the degree of conversion of lactose to lactobionate acid. Further, use of a catalase decrease the amount of H_2O_2.

Accordingly, a separate aspect of the invention relates to a method for the preparation of a composition comprising lactobionic acid comprising

(i) adding to a dairy base a carbohydrate oxidase, capable of converting lactose to lactobionic acid,

(ii) incubating for a suitable time at a suitable temperature in presence of a suitable amount of oxygen to obtain the composition comprising lactobionic acid, and

(iii) optionally, purifying the lactobionic acid in a suitable way to get a composition comprising lactobionic acid with a desired degree of lactobionic acid purity,

wherein there is also added a catalase in the method in an amount that decrease the amount of H_2O_2.

As described above, when the carbohydrate oxidase converts lactose to lactobionic acid there are generated H_2O_2. The reaction scheme may be described as:

$$\text{lactose} + \tfrac{1}{2}O_2 \rightarrow \text{lactobionic acid} + H_2O_2$$

The catalase may be added after the carbohydrate oxidase has reacted to make the lactobionic acid. However, preferably the catalase is added together with the carbohydrate oxidase in step (i).

An advantage of adding also a catalase together with the carbohydrate oxidase in step (i) is that one is able to significantly (up to 50%) reduce the requirement of oxygen and thereby one does not have to supply as mush oxygen e.g. in the form of air. Actually, if one is adding an adequate amount of catalase it is not necessary to supply extra oxygen e.g. in the form of air. The latter

preferably requires also extra addition of a suitable amount of H_2O_2. This extra added H_2O_2 may be based on H_2O_2 from e.g. any suitable commercial source.

Accordingly, a preferred embodiment of this separate aspect and the method of the first aspect of the invention is wherein essentially all of the suitable amount of oxygen required in step (ii) of the method of the separate aspect and the method of the first aspect is obtained by extra addition of a suitable amount of H_2O_2 and wherein the catalase generates the required oxygen by use the available H_2O_2.

The term "essentially all of the suitable amount of oxygen" relates here to that there is enough oxygen for the enzymatic reaction to work adequately and in particular that it is not necessary to actively add extra oxygen during the incubation step e.g. by continuously mixing air into the dairy base under incubation.

A preferred embodiment of this separate aspect is wherein during the incubating the pH is maintained, by adequate addition of a base, at a pH from 4 to 8 for a time period that is sufficient long to get a degree of conversion of lactose to lactobionic acid that is at least 2.5% higher than in a comparative control method where the only comparative difference is that during the incubation the pH is not maintained by adequate addition of a base.

All embodiments, as described herein, with respect to the method of the first aspect of the present invention are also preferred embodiments with respect to the method of this separate aspect of the invention. This is particular relates to the preferred embodiment wherein the dairy base is milk and the base is $Ca(OH)_2$.

Further, all embodiments, as described herein, with respect to the method of the separate aspect of the present invention, are also preferred embodiments with respect to the method of the first aspect of the invention.

In a preferred embodiment in step (i) of the method of the first aspect of the present invention and the corresponding embodiments thereof is preferably also added a catalase in an amount that decrease the amount of H_2O_2.

Preferably, the catalase is added in an amount that also improves the degree of conversion of lactose to lactobionate acid.

A number of suitable catalases are known to the skilled person. For instance, the commercial available catalase Catazyme® from Novozymes A/S.

The amount of catalase added to the method as described herein will generally depend to the amount of H_2O_2 one wants to have in the final dairy product. Accordingly, depending on the particular dairy product of interest, especially if the dairy product is milk, the amount of catalase added to the method as described herein, is an amount that is sufficient big to get an at least 10% decrease in the amount of H_2O_2 as compared to a comparative control method where the only comparative difference is that catalase is not added.

More preferably, the amount of catalase added to the method as described herein, is an amount that is sufficient big to get an at least 25% decrease in the amount of H_2O_2 as compared to a comparative control method where the only comparative difference is that catalase is not added, even more preferably the amount of catalase added to the method as described herein, is an amount that is sufficient big to get an at least 75% decrease in the amount of H_2O_2 as compared to a comparative control method where the only comparative difference is that catalase is not added.

The comparative control method should, except for the non-addition of the catalase, be performed identically (same oxidase in same amount, same dairy base, same incubation time, temperature, presence of oxygen and addition of base and etc.) to the method for improving lactobionic acid as described herein. This is in accordance with the normal understanding of a control as used herein, because the control is made to identify the positive effect of the addition of catalase.

Extra purification of the lactobionic acid:

Optionally, it is possible to purify the lactobionic acid in a suitable way to get a composition comprising lactobionic acid with a desired degree of lactobionic acid purity.

The skilled person knows how to do that and depending on the specific needs of interest a composition comprising at least 30% lactobionic acid, or at least 90% lactobionic acid may be obtained.

Suitable ways of purifying the lactobionic acid are filtration, ion exchange, concentration and drying.

A composition comprising lactobionic acid may be used e.g. as a food additive or ingredient in a way known in the art.

Starter culture comprising lactic acid bacteria:

In general, extra elements or ingredients may be included in the method as described herein in agreement with specific needs. The skilled person known is aware of numerous of such extra elements or ingredients.

In a preferred embodiment a starter culture comprising lactic acid bacteria may be included in the method as described herein. The starter culture may be added to the diary base before or after the oxidase is added to the base. This will depend on the specific fermentation profile one is interested in.

The term "lactic acid bacteria" denotes herein a group of Gram-positive, non-sporing bacteria, which carry out a lactic acid fermentation of sugars.

Among others, it includes species of lactic acid bacteria belonging to genus *Lactobacillus*, such as *Lactobacillus helveticus*, *Lactobacillus delbruckii subsp. bulgaricus*, etc., lactic acid bacteria belonging to genus *Lactococcus*, such as *Lactococcus lactis*, lactic acid bacteria belonging to genus *Streptococcus*, such as *Streptococcus salivarius*, lactic acid bacteria belonging to genus *Leuconostoc*, such as *Leuconostoc lactis*, lactic acid bacteria belonging to genus *Bifidobacterium*, such as *Bifidobacterium longuni* or *Bifidobacterium. breve*, and lactic acid bacteria belonging to genus *Pediococcus*.

The lactic acid bacteria may be used as a mixture with other microorganisms, e.g. yeasts.

A process for making a dairy product

As described above, numerous suitable uses of a composition comprising lactobionic acid as described herein to make a dairy product of interest is known to the skilled person and reference is e.g. made to the prior art publications discussed above. Accordingly, once a composition comprising lactobionic acid is made by the method as described herein this composition may be used in any suitable way to make a dairy product of interest.

The term "dairy product" is to be understood as any dairy product including a dairy base, as defined above. Examples of dairy products applicable for the present invention are products like yoghurt, milk such as e.g. a calcium fortified milk and cheese such as process cheese (e.g. for pizza), cream cheese and cottage cheese.

EXAMPLES:

Example 1:

In a large scale trial with 400kg substrate solution consisting of 6% whey permeate powder (from EPI, France). Temperature was adjusted to 49°C.
Carbohydrate oxidase enzyme dosage corresponding to 40000 OXU in 400Litre solution = 1250g enzyme. The enzyme was achieved from *Microdochium nivale* as described in patent application WO 9931990. A Oxidase Unit (OXU) is basically defined as the amount of enzyme that oxidizes one µmol lactose per minute under the here stated conditions. Here is one OXU defined as one mg of pure lactose oxidase enzyme - relative to an enzyme standard.

Reaction was followed by pH and oxygen sensor. Air was mixed into the substrate by recirculation of substrate to the tank utilizing the pump turbulence (Landia pump 5.5kWh). When oxygen has returned to >90% the reaction is finished and the product was heated to 85°C for enzyme inactivation. Samples were taken during the reaction, and were heated to 85°C for inactivation of the enzyme.
pH in substrate solution before enzyme addition was 6.52. After termination (5 hours reaction time) of the reaction pH had decreased to 3.62. Oxygen content in substrate was 5.82mg/L at

time = 0 and reduced within 5 minutes after enzyme addition to less than 0.5mg/L. Data from the trial is seen in the table below.

Time, minutes	pH	Oxygen
0	6,52	5,82
5	6,39	0,50
15	6,02	0,49
30	5,63	0,33
60	5,12	0,40
65	5,02	0,40
90	4,82	0,42
120	4,46	0,32
175	4,00	0,12
240	3,70	0,90
250	3,66	1,70
260	3,64	3,66
270	3,64	4,00
280	3,64	4,45
290	3,62	5,45
300	3,62	5,52

Degree of conversion of lactose to lactobionic acid was 41%.

Example 2

166 kg substrate similar to the one described in example 1 was added carbohydrate oxidase 833g (corresponding to 26700 OXU). The oxidase used was the same as in example 1. pH was held in the range 5 – 6 by addition of 5N NaOH solution. When oxygen level returned to initial level (after 5 hours) a total amount of 3910 mL 5N NaOH had been used for pH-control. This amount corresponds to the conversion of > 90% lactose to lactobionate according to the reaction scheme:

lactose + $\frac{1}{2}O_2$ → lactobionic acid + H_2O_2 , where the NaOH is balance the acid formed.

Example 3

166 kg substrate similar to the one described in example 1 was added carbohydrate oxidase 441g (corresponding to 14100 OXU) and Catalase 25L dosage 39g. The oxidase used was the same as in example 1. The Catalase was Catazyme® from Novozymes A/S.

pH was held in the range 5 – 6 by addition of 5N NaOH solution. When oxygen level returned to initial level (after 4 hours 40 minutes) a total amount of 4860g 5N NaOH had been used for pH-control. This amount corresponds to the conversion of 100% lactose to lactobionate acid.

Example 4:

Objective

The objective was to test the concept of producing Ca-fortified milk by lactose oxidase catalysed reaction in milk by keeping pH constant with Ca(OH)2 addition. Samples were taken at different base additions and the milk is heat treated and evaluated (taste and stability).

Method

Substrate skim milk 1.5kg

Temperature 50C

Fresh air is flushed over the surface of the stirred substrate.

Enzyme was carbohydrate oxidase preparation from *M.nivale* dosage 0.085OXU/g solution = 3.98g. The oxidase used was the same as in example 1.

Catalase 25L dosage 0.5g (Catazyme® from Novozymes A/S).

The oxidase and catalase was added to the substrate and incubated as described below.

pH is kept constant by addition of 1N $Ca(OH)_2$

Samples of 100mL were taken after base consumption of 22.2mL and 41.15mL, and heat treated 85°C for 15 minutes.

After cooling the samples were evaluated with respect to taste and stability.

Results

pH in skim milk is 6.65 at 50°C, which was used as the set-point in the pH-stat titration unit.

pH in the samples were 6.76 and 6.88 measured at room temperature.

None of the samples were grainy or had any sign of sediment. The last sample seemed slightly more viscous. Taste was good for both samples and no off flavour was detected.

The Ca-level in the milk after the $Ca(OH)_2$ addition was increased:

Sample 1: 48%

Sample 2: 88%

The degree of conversion of lactose to LBA estimated from the amount of base used was:

Sample 1: 22.2mL corresponds to 11% conversion

Sample 2: 41.15mL corresponds to 20% conversion

Conclusion

This example demonstrates that a Ca-fortified milk without taste defects can be produced by addition of $Ca(OH)_2$ during enzymatic oxidation of lactose to lactobionic acid as long as the addition of base is done in a titration equipment keeping pH constant. Ca-addition up to 88% extra in the skim milk was obtained. This level by far exceeds the level normally aimed at in milk drinks.

CLAIMS

1. A method for the preparation of a composition comprising lactobionic acid comprising
(i) adding to a dairy base a carbohydrate oxidase, capable of converting lactose to lactobionic acid,
(ii) incubating for a suitable time at a suitable temperature in presence of a suitable amount of oxygen to obtain the composition comprising lactobionic acid, and
(iii) optionally, purifying the lactobionic acid in a suitable way to get a composition comprising lactobionic acid with a desired degree of lactobionic acid purity,
characterized in that during the incubating the pH is maintained, by adequate addition of a base, at a pH from 5 to 6.9.

2. The method of claim 1, wherein the adequate addition of a base is done for a time period that is sufficient long to get a degree of conversion of lactose to lactobionic acid that is at least 2.5 % higher than in a comparative control method where the only comparative difference is that during the incubation the pH is not maintained by adequate addition of a base.

3. The method of claims 1 or 2, wherein the dairy base is milk or more preferably whey or fractions of whey.

4. The method of any of the preceding claims, wherein the carbohydrate oxidase is a microbial carbohydrate oxidase.

5. The method of claim 4, wherein the carbohydrate oxidase is a carbohydrate oxidase obtained from a fungus belonging to the genus *Microdochium*, more preferably wherein the fungus is *Microdochium nivale* and even more preferably wherein the fungus is *Microdochium nivale* CBS 100236.

6. The method of any of the preceding claims, wherein the there is used an amount of oxidase that is from 0.1 to 1000 OXU per kg of dairy base, more preferably from 1 to 500 OXU per kg of dairy base, and even more preferably from 5 to 100 OXU per kg of dairy base.

7. The method of any of the preceding claims, wherein the suitable temperature of step (ii) is from 0°C to 80°C.

8. The method of any of the preceding claims, wherein the base is $Ca(OH)_2$

9. The method of any of the preceding claims, wherein during the incubating (of step (ii)) the pH is maintained, by adequate addition of a base, at a pH from 5 to 6.9 for a time period that is sufficient long to get a degree of conversion of lactose to lactobionic acid that is at least 5% higher than in a comparative control method where the only comparative difference is that during the incubation the pH is not maintained by adequate addition of a base, more preferably at least 15% higher than in the comparative control method, even more preferably at least 30% higher than in the comparative control method and most preferably at least 45% higher than in the comparative control method.

10. The method of any of the preceding claims, wherein the pH is maintained, by adequate addition of a base, at a pH from 5.5 to 6.9, more preferably at a pH from 6 to 6.9.

11. The method of any of the preceding claims, wherein the pH is maintained at the stable pH from the start of the enzymatic reaction that is converting lactose to lactobionic acid.

12. The method of any of the preceding claims, wherein the pH is maintained at the stable pH level for a time period that at least last until the oxygen level of the incubated dairy base has returned to more than 90% of the initial level.

13. The method of any of the preceding claims, wherein the pH is maintained at the stable pH level as described herein for a time period from 30 minutes to 35 hours, more preferably from 1 hour to 20 hours and even more preferably from 2 hours to 12 hours.

14. The method of any of the preceding claims, wherein in step (i) of the method of claim 1 is also added a catalase in an amount that decreases the amount of H_2O_2.

15. The method of claim 14, wherein the amount of catalase added is an amount that is sufficient big to get an at least 10% decrease in the amount of H_2O_2 as compared to a comparative control method where the only comparative difference is that catalase is not added.

16. The method of claims 14 or 15, wherein essentially all of the suitable amount of oxygen required in step (ii) of claim 1 is obtained by extra addition of a suitable amount of H_2O_2 and wherein the catalase generates the required oxygen from the available H_2O_2.

17. The method of any of the preceding claims, wherein by making purification according to step (iii) of claim 1 is obtained a composition comprising at least 30% lactobionic acid or at least 90% lactobionic acid.

18. The method of any of the preceding claims, wherein a starter culture comprising lactic acid bacteria is included in the method of the preceding claims and wherein the starter culture may be added to the diary base before or after the oxidase is added to the dairy base.

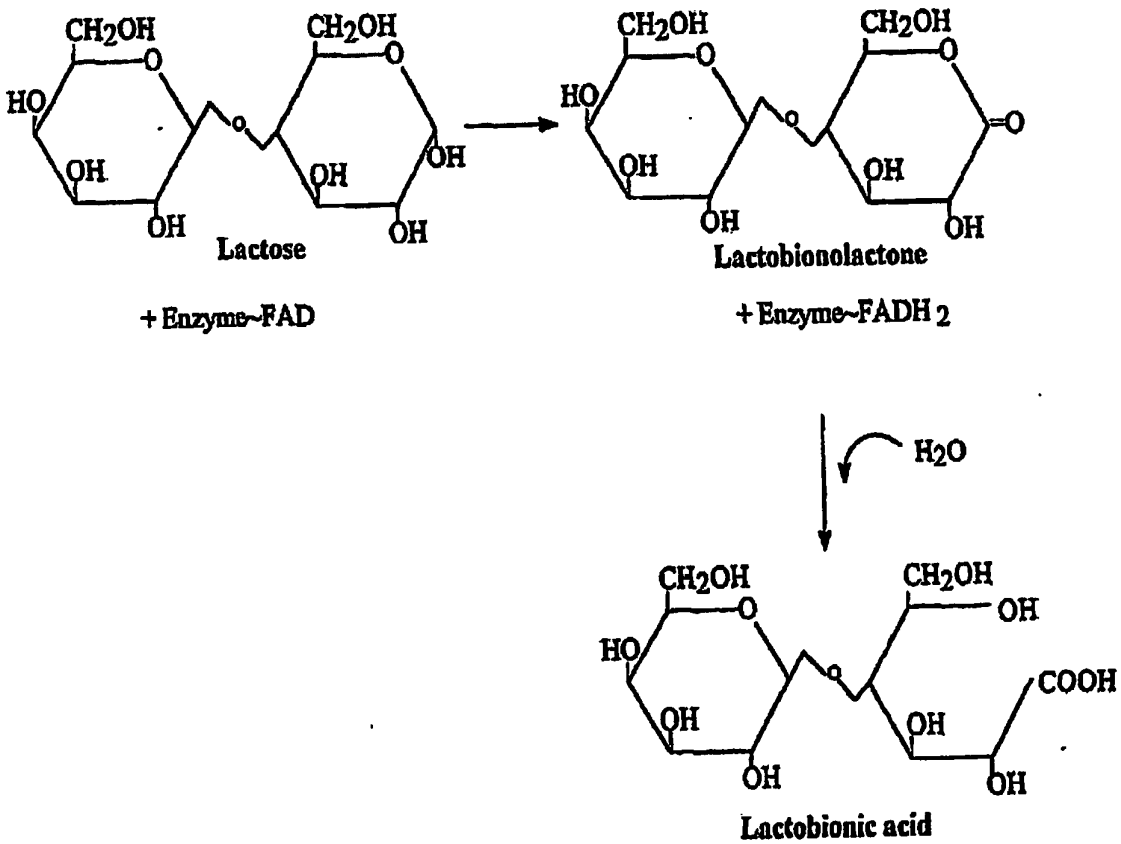
19. A process for making a dairy product comprising first to make a composition comprising lactobionic acid according to a method for making such a composition according to any of claim 1 to 17 and then use this composition to make the dairy product.

20. The process of claim 19, wherein the dairy product is a dairy product selected form the group consisting of a yoghurt, a milk such as e.g. a calcium fortified milk and a cheese such as process cheese (e.g. for pizza), cream cheese and cottage cheese.

ABSTRACT

A method for getting increased yields of lactobionic acid comprising adding to a dairy base, such as milk or whey, a carbohydrate oxidase, capable of converting lactose to lactobionic acid, wherein the method is performed under stably control of pH from pH 5 to 6.9.

Figure 1



Enzyme Regeneration

Enzyme~FADH 2+ O2 ⟶ Enzyme~FAD + H 2O2

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